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PRESENCE OF IDF45 (mIGFBP-3) BINDING SITES ON CHICK EMBRYO FIBROBLASTS

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SUMMARY : IDF45 (inhibitory diffusible factor) a mouse insulin-like growth factor binding protein (mIGFBP-3) has been shown to 100 percent inhibit DNA synthesis stimulated by serum in chick embryo fibroblasts (CEF). Our previous results suggested that this large inhibition by IDF45 of serum stimulation was not just the result of its inhibitory activity toward IGF present in serum.

The addition of Mn^{2+} (10^{-3}M) in the incubation medium enables us to show the presence of numerous binding sites per cells (about 60,000) of mIGFBP-3. However the dissociation constant (10^{-8}M) indicated that this mouse IGFBP-3 bound to the membrane with low affinity.

These findings lend new support to the assumption of the bifunctional property of IGFBP-3, which would have an effect outside the cell (binding of IGF in the medium) and another effect within cells or on the surface.

IGF binding proteins have been shown to both inhibit and enhance (1,2,3,4,5,6) the stimulation of DNA synthesis induced by IGF-I. The binding of these proteins to the cell membrane is a much debated question. From amniotic fluid, two proteins of Mr 31 kDa were purified: one of them (peak C) inhibited and the other (peak B) enhanced the stimulation of DNA synthesis induced by IGF-I in smooth muscle cells (2). ^{125}I-labelled peak B protein, but not peak C protein, was shown to bind the muscle cells and the radioactivity bound was decreased in the presence of non-labelled peak B protein (2). Blum et al. did not find specific binding of IGFBP-3 alone or of the IGF-I/IGFBP-3 complex on the BHK21 cell membrane (4). Using cross-link affinity labelling with ^{125}I-IGF-I, the presence of IGFBP-3 on human (7) and bovine fibroblasts (6) was shown, and was suggested as being responsible for the paradoxal increase in ^{125}I-IGF binding to cells as a function of concentration of the non-labelled IGF-I (7,8,9).

From medium conditioned by 3T3 cells, we purified to homogeneity a growth inhibitor, IDF45 (inhibitory diffusible factor of 45 kDa) able both to 100% inhibit serum stimulation of DNA synthesis in chick embryo fibroblasts, and to reversibly prevent the cell growth (10,11). We demonstrated then that IDF45 was an IGF-binding protein (12). Indeed, its N-terminal amino acid sequence is homologous to that of rat IGFBP-3 (13). We previously showed that IDF45 and rat IGFBP-3 have the same biological activities (14), and it is likely that IDF45 and rat IGFBP-3 are similar proteins. In the present publication, we therefore refer to IDF45 as mIGFBP-3 (mouse IGFBP-3). Our previous data suggested that inhibition by IGFBP-3 of serum stimulation was not just the result of its inhibitory activity toward IGF present in the serum (12, 14). Therefore, we assumed that IGFBP-3 was bifunctional, able to bind IGF and inhibit stimulation of DNA synthesis induced by this hormone, but also to inhibit serum growth factors different from IGF-I and -II.

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We show here that mIGFBP-3, in the presence of Mn^{2+}, binds to the membrane of chick embryo fibroblast, but with low affinity. mIGFBP-3 was also able to enter the cells. The presence of mIGFBP-3 binding sites in the membrane of chick embryo fibroblasts as well as mIGFBP-3 internalization, support the hypothesis of bifunctional IGFBP-3 activity.

MATERIALS AND METHODS

Cells - Primary cultures of chicken embryo fibroblasts (CEF) were prepared from 10-day old Leghorn chick embryos as described (15).

mIGFBP-3 iodination - mIGFBP-3 was purified to homogeneity from medium conditioned by dense cultures of 3T3 Swiss cells (11).
In the first experiment (table 1), 1.5 µg pure mIGFBP-3 was iodinated with 1mCi of carrier-free $Na^{125}I$ (IMS-30, Amersham International) by the lactoperoxidase/glucose oxidase method (Enzymobeads, Bio Rad) following manufacturer's direction. In the other experiments, 1.5 µg of pure mIGFBP-3 were iodinated with 1mCi $Na^{125}I$ by the chloramine T method (16). After iodination ^{125}I-mIGFBP-3 was chromatographed on a column (0.55 x 8.5 cm) of BioGel P100 (Bio Rad), equilibrated and eluted with 0.1 M acetic acid, 0.25 M NaCl pH 3.0 containing 0.1 mg/ml of bovine serum albumin (BSA).
The inhibitory activity of ^{125}I-mIGFBP-3 was verified.

Binding studies - Secondary cultures of CEF were seeded at 4.8 x 10^5 cells/well in Eagle medium with 5% newborn calf serum in 24 well culture plates, 5 h later medium was discarded and cells were maintained for 42 h in serum-free medium. Then cells were washed three times with binding buffer (Hanks saline medium containing 25 mM $NaHCO_3$, 25 mM Hepes and 0.25% BSA pH 7.4) and were incubated at room temperature in 0.125ml of this buffer with ^{125}I-IGFBP-3 in conditions described in legends.

After incubation, cells were washed at 4°C twice with 1ml of cold PBS containing 0.25% BSA before addition for 3 min of an acidic solution (0.1 M acetic acid, 0.5 M NaCl). Radioactivity of the acidic solution and cell proteins solubilized in NaOH (0.6 N) were counted in a gamma counter (Beckman 4000).

Protein determination in each well was performed using the Lowry method with BSA as standard. Data are the mean of three determinations (±SD). The affinity of mIGFBP-3 with CEF membrane was determined using the LIGAND program.

Menbrane-enriched preparations were prepared from secondary cultures of CEF using method described (17).

CEF membrane-enriched preparations (150 µg) in microfuges were washed twice with cold 10 mM Hepes pH 7.0 by centrifugation at 4°C 12,000 x g 15 min, then incubated 90 min at room temperature in 150ml of binding buffer.

At the end of the incubation period, membranes were centrifuged for 15 min, 12,000 x g at 4°C. Pellet was resuspended in 10 mM Tris, 0.25 M sucrose pH 7.4 and washed twice with this buffer by centrifugation in the same condition. The resulting pellet was solubilized in solution A (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, 3% (w/v) octylglucoside) for 10 min at 4°C and mixed with a concentrated solution of SDS buffer according to Laemmli (18) to a final concentration of 2.3% SDS.

Electrophoresis and autoradiography - SDS-PAGE were performed on PhastGel 10-15% gradient or 7.5% homogeneous using the Phastsystem (Pharmacia-LKB) according to the manufacture's instructions, with radioactive standard proteins purchased from Du Pont-New England Nuclear: myosin, lactoglobulin A, carbonic anhydrase, ovalbumin, albumin, phosphorylase b, [methyl-^{14}C]methylated.

After electrophoresis, PhastGels were fixed in 10% acetic acid, 5% glycerol and dried. Autoradiography was performed using Hyperfilm MP (Amersham International) and Dupont Lightning Plus screens.

RESULTS

mIGFBP-3 internalization

In the first experiment (table 1), cells were incubated with ^{125}I-mIGFBP-3, washed and treated with 0.1 M acetic acid, 0.5 M NaCl pH 3.0 to separate the ligand bound to the membrane from the cells. Then cells were solubilized in NaOH 0.6 N.

Table 1

Binding of ^{125}I-mIGFBP-3 to CEF

unlabelled mIGFBP-3	^{125}I-mIGFBP-3 (cpm/well) in acid fraction	in NaOH fraction
0	187 ± 17	454 ± 26
200 ng/ml	188 ± 30	320 ± 1
2000 ng/ml	212 ± 7	187 ± 9

Cells were incubated for 2 h with 8 ng/ml ^{125}I-mIGFBP-3 (specific activity : 20,000 cpm/ng) in binding buffer in the absence or presence of unlabelled mIGFBP-3.

Results showed that while the radioactivity bound on the membrane represented only a small percent of the total radioactivity added and did not decrease when incubated with large concentrations of unlabelled mIGFBP-3, radioactivity found in the cells (NaOH fraction) decreased in the presence of unlabelled mIGFBP-3. This first experiment suggested that mIGFBP-3 was internalized; however, we were unable to show the presence of specific binding sites. We assumed that this was due to the poor affinity of mIGFBP-3 for the cell.

In the experiments which followed, we tried to increase the stability of mIGFBP-3 binding by incubating cells with Mn^{2+}, known to stabilize interactions between proteins of the extra cellular matrix and integrins (19,20,21).

Mn^{2+} effect on the binding of mIGFBP-3

In the experiment described in fig 1, cells were incubated with ^{125}I-mIGFBP-3 and different concentrations of $MnCl_2$. Since these experiments required a large quantity of non-labelled mIGFBP-3, we determined the non specific bound radioactivity by using mouse serum which contains mIGFBP-3.

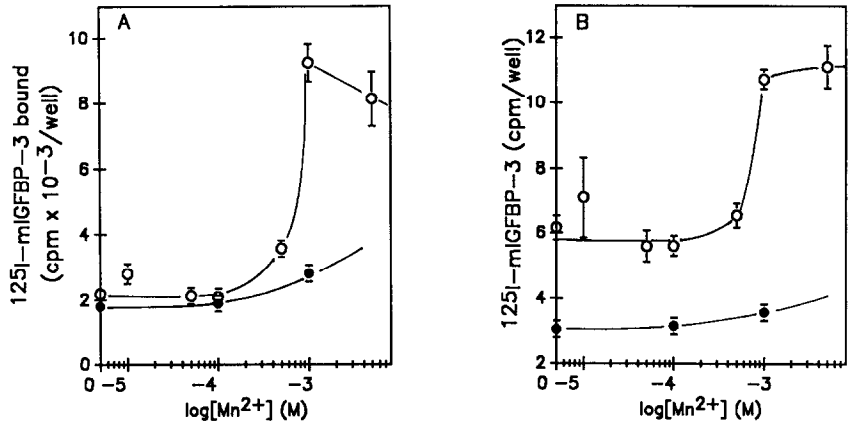


Fig. 1 . Manganese effect on ^{125}I-mIGFBP-3 binding : dose-response curve.
Cells were incubated for 2 h with 10 ng/ml ^{125}I-mIGFBP-3 (specific activity: 60,000 cpm/ng) alone (-○-) or with 10% (v/v) mouse serum (-●-) in binding buffer containing different concentrations of $MnCl_2$. Cpm in acid solution (A), cpm in NaOH solution (B).

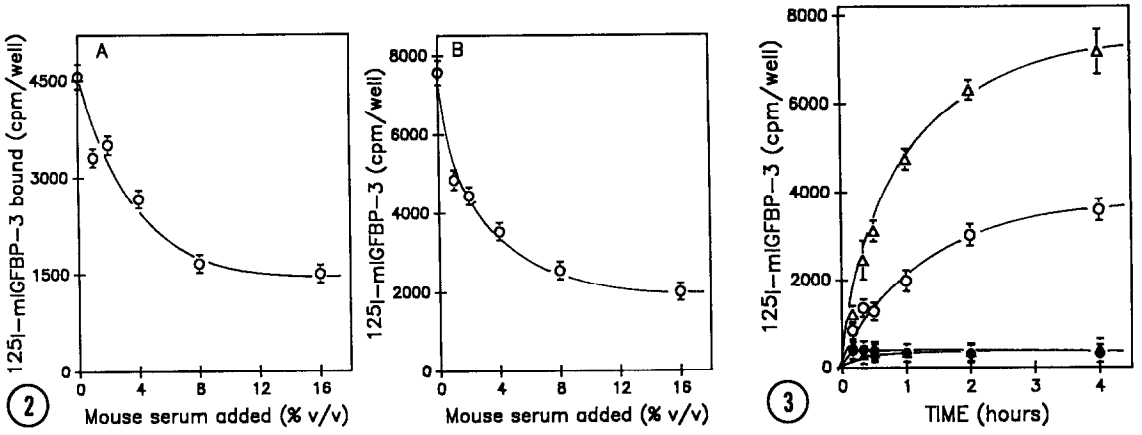


Fig. 2 . Serum effect on ^{125}I-mIGFBP-3 binding : dose-response curve.
Cells were incubated for 2 h in binding buffer supplemented with 10^{-3} M $MnCl_2$, in the presence of 10 ng/ml ^{125}I-mIGFBP-3 (specific activity: 60,000 cpm/ng) and increasing concentrations of mouse serum. (A), radioactivity in the acid solution. (B), radioactivity in cells solubilized in NaOH.

Fig. 3 . Time-course of ^{125}I-mIGFBP-3 binding.
Cells were incubated at different times in binding buffer with 10 ng/ml ^{125}I-mIGFBP-3 (specific activity: 60,000 cpm/ng)in the presence or absence of 10% (v/v) mouse serum. Radioactivity in the acid solution : (-○-) ^{125}I-mIGFBP-3 alone; (-●-) in the presence of serum. Radioactivity in cells solubilized in NaOH : (-△-) ^{125}I-mIGFBP-3 alone; (-▲-) in the presence of serum.

The radioactivity bound to the membrane was 4-fold increased in the presence of 10^{-3} M Mn^{2+} (fig 1A). The radioactivity found in the cell was only two-fold increased in the presence of Mn^{2+} (fig 1B). It is of interest that mouse serum specifically decreased Mn^{2+} induced stimulation of ^{125}I-IGFBP-3 binding to the cell membrane.

Fig 2 shows that the competition by serum was dose-dependent; the maximum decreases in ^{125}I-mIGFBP-3 bound to the cells (fig 2A) or in the cells (fig 2B) were observed with 10% serum. Therefore, in the next experiments, incubation was performed in the presence of 10^{-3} M Mn^{2+} which gives maximum binding, and non-specific binding was determined in the presence of 10% mouse serum.

Kinetics of ^{125}I-mIGFBP-3 binding

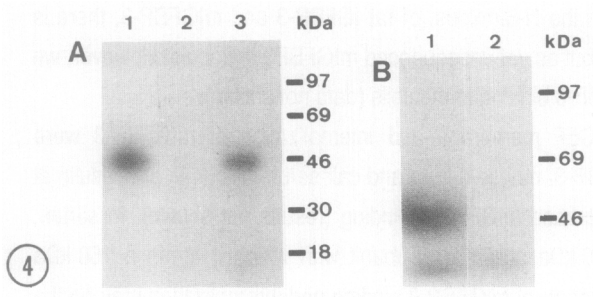
In the experiment described in fig 3, cells were incubated at different times in the presence of ^{125}I-mIGFBP-3. We observed that at 20°C, equilibrium for the binding of mIGFBP-3 to cell membranes and internalization was reached in 2-3 h.

In the presence of 10% mouse serum the radioactivities in the acetic acid and NaOH fractions were low, and were independent of the incubation time, again suggesting that it was non-specific radioactivity binding.

SDS-PAGE of proteins of labelled cells

A fraction enriched in membranes was prepared from CEF. Cells or membrane-enriched preparations were incubated with ^{125}I-mIGFBP-3 in the presence or absence of 10% mouse serum. Then they were washed and solubilized in SDS. The proteins were submitted to electrophoresis.

Autoradiograms of SDS-PAGE showed radioactivity at the 45 kDa and that serum decreased the radioactivity found in this band (fig 4A and 4B). In the same experiment, cells or membrane-enriched preparations were incubated with ^{125}I-IGFBP-3 and cross-linked with 0.25 mM DSS (disuccinimidyl



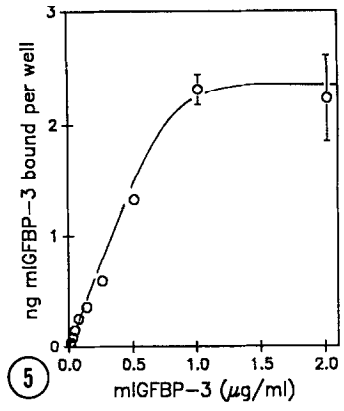


Fig. 4 . (A) : Binding and internalization of ^{125}I-mIGFBP-3 : Internalization in CEF.
Cells were incubated for 2 h in binding buffer containing 10^{-3} M $MnCl_2$ with 10 ng/ml ^{125}I-IGFBP-3 (specific activity: 100,000 cpm/ng) alone or in the presence of 10% (v/v) mouse serum. After washing as described in Materials and Methods, cells were solubilized in SDS, submitted to SDS-PAGE on PhastGel gradient 10-15% and autoradiographed (7 days). (1) control ^{125}I-IGFBP-3 without cells; (2) in the presence or (3) in the absence of serum.

(B) : Binding to CEF membrane.
Cell membranes were incubated for 90 min as described in (A). Then they were washed, solubilized in SDS, submitted to SDS-PAGE on homogenous PhastGel 7.5% and autoradiographed (7 days). (1) ^{125}I-IGFBP-3 alone; (2) in the presence of 10% (v/v) mouse serum.

Fig. 5 . Binding site number.
Cells were incubated for 2 h in binding buffer containing 10^{-3} M $MnCl_2$ with 10 ng/ml ^{125}I-mIGFBP-3 (specific activity: 60,000 cpm/ng) and increasing concentrations of unlabelled mIGFBP-3. mIGFBP-3 bound to cells (ng) were calculated from cpm bound in acid solution and the mIGFBP-3 specific radioactivity at each point bound to cell membrane.

suberate) before SDS PAGE. The radioactivity was found in the stacking gel even when the proteins were solubilized in different detergent (data not shown).

Determination of dissociation constant and the number of binding sites

Cells were incubated with 10 ng/ml of ^{125}I-mIGFBP-3 in the presence of increasing concentrations of unlabelled mIGFBP-3.

Radioactivity bound to the cells (detachable in acid medium) was determined. mIGFBP-3 which bound to the cell membrane as a function of mIGFBP-3 concentration was calculated taking account of the specific radioactivity of mIGFBP-3 (fig 5). Saturation was obtained at a concentration of 1 μg/ml mIGFBP-3. At this concentration 2.2 ng mIGFBP-3 were bound to 5×10^5 cells (84 μg proteins). This permitted an evaluation of 60,000 binding sites/cells. Analysis of radioactive ^{125}I-mIGFBP-3 binding to the cell membrane on the computer program LIGAND in the displacement mode evaluated a dissociation constant of about 10^{-8} M. Therefore, mIGFBP-3 bound to the cell membrane with low affinity, but at numerous binding sites.

DISCUSSION

As stated in the Introduction, the binding of IGFBP to the cell membrane is subject to controversy. Our first experiment (table 1) pointed out the difficulty of demonstrating the binding of mIGFBP-3 to the cell membrane in the absence of Mn^{2+}. This cation, at the concentration of 10^{-3} M, is known to stabilize the low affinity interaction between fibronectin and integrins. At this concentration,

Mn^{2+} was also able to stabilize the interaction of mIGFBP-3 with its binding site on the CEF membrane, enabling the evaluation of the number of binding sites per cells. The affinity of mIGFBP-3 for its binding site is weak, like that of fibronectin for its cellular receptor (22,23). Fibronectin binds to integrins via the RGD sequence. Because of the homology at the N-terminus, of rat IGFBP-3 and mIGFBP-3, there is probably no RGD sequence in the remaining but as yet unsequenced mIGFBP3 molecule. However, we verified that fibronectin did not prevent mIGFBP-3 binding to the cells (data not shown).

The binding of mIGFBP-3 to the CEF membrane and internalization of mIGFBP-3 were specifically decreased by non-labelled mIGFBP-3, mouse serum and calf serum. Albumin and insulin at the same concentrations were unable to decrease mIGFBP-3 binding (results not shown). In serum, IGFBP-3 binds mainly to IGF, and to an 80 kDa acid-labile subunit with which it forms a 150 kDa complex (24,25). Therefore the inhibition by serum of mIGFBP-3 binding and internalization may be the result of an exchange between ^{125}I-mIGFBP-3 and non-labelled mIGFBP-3 in serum: ^{125}I-mIGFBP-3 would be trapped in the 150 kDa complex.

In a previous work, we observed that mIGFBP-3/IDF45 was able to inhibit the early stimulation of RNA synthesis induced by serum. However inhibition was greater when cells were preincubated with mIGFBP-3 before addition of serum (26). Our present data may explain this observation. Indeed, since internalization of mIGFBP-3 appeared greater in the absence than in the presence of serum, preincubation with mIGFBP-3 may be required for mIGFBP-3 to enter the cells so as to inhibit some serum-induced stimulatory events. By contrast, inhibition by mIGFBP-3 of RNA synthesis induced by IGF-I did not require preincubation (26), since this inhibition was the result of IGF-I binding with IGFBP-3 outside the cells.

We did not succeed in characterizing the IGFBP-3 binding site on the membrane by cross-link affinity labelling; another must be found to characterize the molecule.

In conclusion, our results indicate the presence on CEF membranes of binding sites of mIGFBP-3. However, binding of mIGFBP-3 was of low affinity and required Mn^{2+}. These findings lend new support to the assumption of the bifunctional property of IGFBP-3, which would have an effect outside the cells (binding of IGF in the medium) and another effect within cells or on the cell surface.

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